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The expression of the gene *asebia* in the laboratory mouse

3. Sebaceous glands

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SUMMARY

Contrary to what their name *asebia* implies, mice homozygous for the *ab* gene do possess actively secreting sebaceous glands which develop normally from the follicular outer root sheath, at 18 days post-conception. However, by the 20th day post-conception, these mice exhibit the abnormal sebaceous cytodifferentiation which remains typical of the asebic glands throughout life. Nests of outer root sheath cells below the sebaceous glands also undergo atypical sebaceous differentiation. The smooth membrane system and mitochondria, which appear to be responsible for the orderly accumulation of lipid droplets and sebum production in normal mice, become increasingly abnormal in asebic mice. Fewer lipid droplets form, the smooth endoplasmic reticulum becomes distorted and dilated, and the normal transformation of mitochondria does not occur. Atypical differentiation occurs randomly and 'differentiated' cells often degenerate within the asebic sebaceous glands. Of the larger specialized sebaceous-type glands studied, only the Meibomian glands are similarly affected by the *asebia* mutation, while the anal and preputial glands appear to undergo a more normal cytological differentiation. The abnormalities seen in the asebic sebaceous glands seem to be due to defective regulation of the synthetic or degenerative processes necessary for completion of normal sebum production. Both the defects of sebaceous glands and the unusual characteristics of the epidermis and hair follicles in asebic mice may be initiated by the abnormal underlying dermis or the apparently abnormal endocrine system.

1. INTRODUCTION

Like many other organs, the sebaceous gland of the mouse passes through a series of phases during which it is subject to different controls. First, the rudiment appears during foetal life as a group of relatively undifferentiated epithelial cells, from the outer root sheath of a hair follicle (Hardy, 1949). Secondly, the rudiment grows and its central cells begin to fill with lipid droplets. Thirdly,

during secretory activity, the lipid-filled cells degenerate and rupture, and the lipid material and cell debris pass out through the hair canal as sebum (Montagna & Parakkal, 1974). From work by Gomot (1959) with the sebaceous-type uropygeal glands of the duck it would seem that an inductive stimulus from the dermis is necessary for the formation of a sebaceous gland rudiment. The second phase of sebaceous cell differentiation may be dependent on a different inductive stimulus from the dermis or mesenchyme, at least in the case of the preputial gland of the mouse (Cunha, 1972a; 1972b). In the third phase, the rate of sebum flow is regulated by the mitotic activity of peripheral cells and the rate of intracellular lipid synthesis both in rats (Ebling, 1967) and in mice (Hamilton, 1974), with peripheral blood androgen and oestrogen levels acting as modulators (Ebling, 1963; 1967; 1973). Bullough & Laurence (1970) have also found that the skin of mice contains a sebaceous gland chalone or mitotic inhibitor, dependent on adrenaline and hydrocortisone, which regulates sebaceous gland activity.

Gates & Karasek (1965) reported that sebaceous glands were completely absent from the skin of adult mice homozygous for the gene *asebia*. In this third paper of the series describing the skin of asebic mice it will be shown that, on the contrary, sebaceous gland rudiments of these mice passed through the first phase and entered the second phase, but that the cell differentiation leading to sebum production was distinctly abnormal. These results and those presented in the two earlier papers (Josefowicz & Hardy, 1978a, 1978b) are discussed in relation to the search for the primary action of this gene.

2. MATERIALS AND METHODS

The mice used in this study were of the BALB/cCrglGa substrain and were described in a previous paper (Josefowicz & Hardy, 1978a). Skin samples from the mid-dorsal region of the abdomen from approximately 80 homozygotes (*ab/ab*), 60 heterozygotes (+/*ab*) and 20 homozygous normal mice (+/+) were examined with the light microscope. The male and female mice examined ranged from 16 days post-conception to approximately 2 years post-natal. Most comparisons were made between homozygous asebic and heterozygous mice, since the latter were indistinguishable from normal mice. Two male litter-mates 8 days old and two male litter-mates 30 days of age provided samples of mid-dorsal skin for electron microscopy. One member of each pair was *ab/ab* the other +/*ab*. All procedures for preparation and staining of sections for light and electron microscopy were described in the previous paper (Josefowicz & Hardy, 1978a).

Twelve *ab/ab* and twelve +/*ab* mature mice were selected from the larger group for a study of the specialized sebaceous glands of the eyelid (Meibomian or tarsal glands), the anal region (anal glands) and the external genital organ (preputial gland of the male and clitoral gland of the female; Hummel, Richardson & Fekete, 1966).

3. RESULTS

(i) *Sebaceous glands of pelage follicles*

The sebaceous gland anlagen developed normally in asebic (*ab/ab*) foetuses at about 18 days post-conception as a bulge of outer root sheath (ORS) cells on the stage 5 pelage hair follicles. However, the glands of mutant foetuses often differentiated from the ORS cells at a deeper level in the follicle than those of their normal litter-mates. By the 20th day post-conception, when the tips of the pelage hairs were about to emerge from some of these follicles, central cells of the sebaceous bulges of normal (+/*ab*, and +/+) foetuses were undergoing typical sebaceous differentiation. However, the cells of asebic mice already appeared to be following an abnormal pathway. Normally differentiating sebaceous cells had spherical nuclei and a cytoplasm filled with distinct lipid droplets which left circular spaces when extracted during histological preparation. These were not seen within the early asebic sebaceous glands. Those cells of asebic glands which were differentiating at all were enlarged, with distorted nuclei and patches of lightly stained cytoplasm (pale pink in haemalum, eosin and picric acid, pale orange in Mallory's triple connective tissue stain).

The abnormalities of the sebaceous glands of the asebic mice became more marked with increasing age. By 5 days after birth, the asebic hair follicles possessed a pair of relatively large single-lobed sebaceous glands which had elongated deep into the dermis. In both the normal and asebic sebaceous glands, cells at all stages of differentiation, as determined by their morphology and staining properties, were present. In the normal (+/*ab*) mice, a progression was evident from the undifferentiated cells at the periphery to the fully differentiated and rupturing cells found where the glands opened into the hair canal. The glands of asebic (*ab/ab*) mice did not possess the same regular organization. Apparently un-differentiated cells were present not only at the periphery but also scattered through the central part of the gland. While 'fully differentiated' cells were usually closer to the hair canal opening, many were also intermingled with cells of varying degrees of differentiation throughout the entire length of the gland (Figs. 1, 2).

The undifferentiated cells of both +/*ab* and *ab/ab* glands were characterized by deeply basophilic nuclei and small amounts of basophilic cytoplasm. The 'differentiating' cells of normal sebaceous glands had rounded nuclei with pale eosinophilic cytoplasm filled with spherical spaces indicating lipid extracted from droplets of uniform size. In the asebic gland, the 'differentiating' cells possessed fewer lipid droplets which were irregular in both size and shape. Measure-ments on electron micrographs showed that these droplets ranged in size from smaller than normal (0·8 μm) to almost five times the normal diameter (4 μm). One micron Epon sections showed 200 or more droplets per section of a sebaceous cell in normal glands, but only 0 to 23 droplets per section of a sebaceous cell in asebic glands. In the light microscope the cytoplasm of the asebic 'differentiating' cells was more lightly stained than that of the undifferentiated gland cells, and

contained spaces of varying size representing extracted lipid material. The resulting irregular 'webbed' appearance (Fig. 2) also prevailed in the 'fully differentiated' asebic sebaceous cells, some of which, unlike those of normal mice which ruptured and dispersed their contents, appeared to remain intact and enclosed by other gland cells. While normal ($+/ab$) sebaceous cells stained progressively less with eosin or with the acid fuchsin component of Mallory's stain as they completed their differentiation (Fig. 3), those of the asebic glands stained more deeply with eosin or acid fuchsin (Fig. 4). The 'fully differentiated' asebic cells stained deeply with the Orange G component of Mallory's stain, which also accentuated a wide cell border.

Electron microscopy showed that the system of smooth endoplasmic reticulum (SER) became increasingly prominent in the cells of normal ($+/ab$) sebaceous glands as they differentiated (Fig. 5). The SER membranes were organized as systems of tubules which appeared to open into the rapidly forming lipid droplets. The tubular interconnections (Fig. 6) seen between droplets suggest that an active redistribution of contents through these connections may be responsible for the relatively uniform size of droplets. Mitochondria were numerous in the earlier stages of differentiation but decreased in number as the SER tubule system became more developed. Coincident with the apparent loss of mitochondria was the appearance of amorphous structures which were closely associated with both the tubules and sebum droplets. Each structure was composed of one or more densely stained granules which were surrounded by a material of lower density. The presence of a series of intermediate forms between intact mitochondria and the above amorphous structures suggested that the latter were derived by degeneration of the former (Fig. 7). It appeared that this 'product' spilled into the adjacent lipid droplets, thus contributing to sebum production (Fig. 8). During the later phases of differentiation the droplets were enclosed by a husk-like membranous structure. The 'husk' could have been formed by a group of tubules wrapping around a droplet and fusing together as they released their products. Bundles of closely packed modified tubules of the SER system

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PLATE 1

Fig. 1. Normal sebaceous glands associated with anagen follicles found in the mid-dorsal skin of a normal $+/ab$ male, 8 days old. 1 μm Epon section stained with Toluidine Blue 0. × 700.

Fig. 2. 'Sebaceous' glands in the mid-dorsal skin of an asebic ab/ab male, 8 days old, revealing the faulty differentiation. Note the variation in lipid droplet (l) size and the webbed appearance (arrow) of may sebaceous cells. 1μm Epon section stained with Toluidine Blue 0. × 700.

Fig. 3. Sebaceous glands from the mid-dorsal skin of a $+/ab$ male, 11 days old. Increased paleness of the sebaceous cell cytoplasm is obvious as differentiation progresses. Mallory's triple connective tissue stain. × 500.
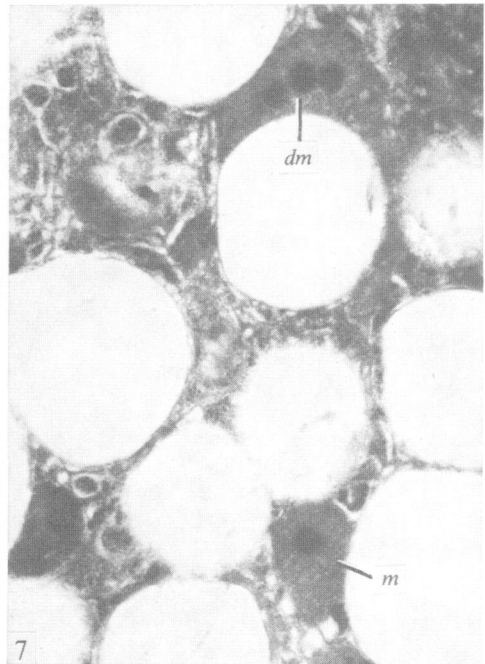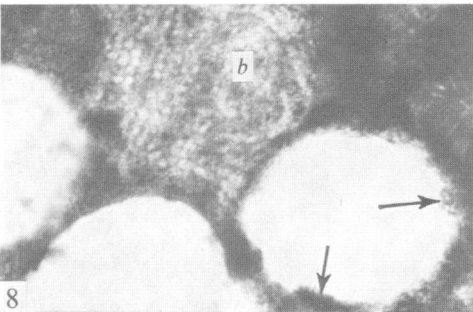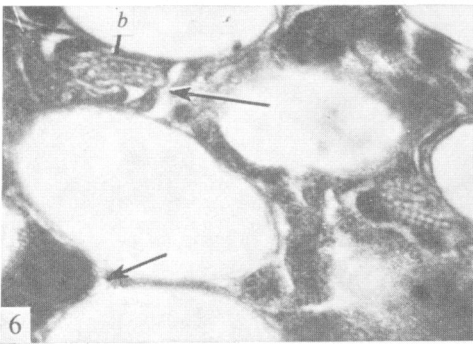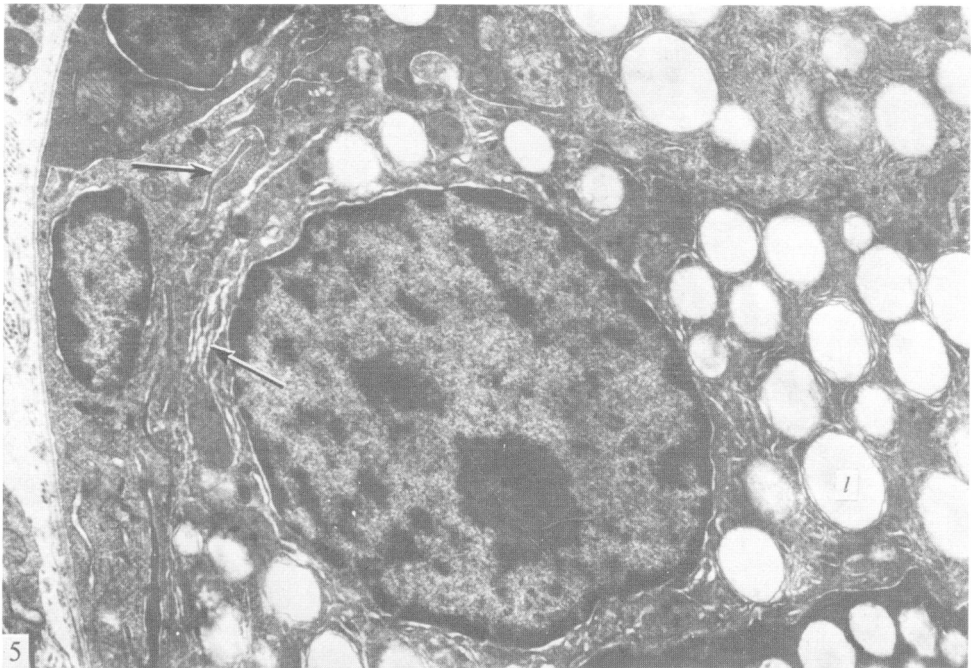
Fig. 4. A pair of sebaceous glands from the mid-dorsal skin of an ab/ab male, 11 days old. Differentiating cells (d) have a somewhat pale cytoplasm while the fully differentiated cells (arrows) have an abnormal 'webbed' morphology and a densely stained cytoplasm. Mallory's triple connective tissue stain. × 500.

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(Figs. 6, 8) were seen in normal sebaceous cells at all stages of differentiation. These bundles varied in number of tubules. Some tubules within the bundles appeared to be connected with typical tubules of SER.

A system of SER tubules was also found in the differentiating cells of asebic (*ab/ab*) glands (Fig. 9) although it differed in several respects. The asebic SER was less extensive than that of the normal mouse and many tubules were dilated and more sharply outlined. Although only a few distinct, irregularly shaped lipid droplets were formed in the asebic cells, they too were associated with the dilated tubule system. The fact that no tubular interconnections were seen suggested an explanation for the irregularities in sebum droplet size. While the asebic sebaceous cells did not have the same dense masses of presumed mitochondrial origin which were typical of normal sebaceous cells, smaller dense granules were seen within the matrices of the still intact mitochondria (Fig. 10). As in the differentiating cells of normal mice, the asebic cells in the early phases of sebaceous differentiation also possessed bundles of modified tubules (Fig. 10). The 'fully differentiated' asebic sebaceous cells having a 'webbed' morphology at the light microscope level were actually cells with their cytoplasm filled with an extensively disrupted tubule and droplet system (Fig. 11). These cells also contained dense masses of amorphous material which may have corresponded to either the mitochondrial masses of the normal cells or to lysosomal material. However, intact mitochondria retaining their smaller dense granules were distinguishable even at this late phase of abnormal differentiation.

An accumulation of acidophilic material, often associated with a large number of nuclear fragments, was commonly observed with the light microscope within the mouth of many hair canals in the mutant mice. This material was not seen in the follicles of the normal animals, and may be attributed, at least in part, to secreted but undistributed sebum produced by the abnormal asebic sebaceous glands.

While it is evident from the above description that sebaceous glands do form from the hair follicles of asebic mice, often these glands tended to differentiate from the outer root sheath cells further down the follicle wall and to form longer

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PLATE 2

Fig. 5. Electron micrograph showing differentiating sebaceous cells from a + /*ab* male, 8 days old. Note the extensive system of SER tubules (arrows) and the forming lipid droplets (*l*). The basal lamina is seen on the left. Uranyl acetate and lead citrate. × 11 500.

Fig. 6. Part of a differentiating sebaceous cell from a + /*ab* male, 8 days old, showing apparent tubular interconnections (arrows) between two lipid droplets. Note the two bundles of modified SER tubules (*b*). Uranyl acetate and lead citrate. × 35 000.

Fig. 7. Part of a differentiating sebaceous cell from a + /*ab* male, 8 days old, illustrating two transitional stages between normal mitochondria and the amorphous structures. The cristae in the lower mitochondrion (*m*) are still discernible, while those in the more degenerated mitochondria (*dm*) cannot be seen. Uranyl acetate and lead citrate. × 27 000.

Fig. 8. Part of a differentiating sebaceous cell from a + /*ab* male, 8 days old, showing the dense 'mitochondrial product' (arrows) at the circumference of a lipid droplet. Also note the large bundle (*b*) of tubules. Uranyl acetate and lead citrate. × 45 000.

sebaceous lobes. Rarely, groups of sebaceous cells, undergoing the previously described type of abnormal differentiation, were seen within the ORS close to the base of the follicle which lay deep in the hypodermis.

The sebaceous glands of older normal mice retained the morphology and activity of young glands. However, those of older asebic mice retained the abnormal sebaceous differentiation (Fig. 12), and frequently changed their morphology. The proliferation of cells both from the outer root sheath and from already differentiating sebaceous glands led to the formation of many irregular and multilobed glands (Figs. 13, 14). The asebic sebaceous glands were usually longer, extending deeper into the dermis than their normal counterparts. Sebaceous differentiation of the outer root sheath cells, extending almost to the base of the follicle, was more frequent in older mice.

(ii) *Specialized sebaceous glands*

In addition to the pair of small single-lobed sebaceous glands associated with each pelage follicle (i.e. the pilosebaceous unit), large specialized sebaceous glands are found in various areas of the mouse skin. Those examined in this study included the Meibomian (or tarsal) glands, each associated with an eyelash but together forming a visible plaque in the subcutaneous tissue of an eyelid; the many multilobed anal sebaceous glands opening into the anal canal; the single pair of large multilobed preputial glands opening into the preputial cavity of the male, and their female counterparts, the clitoral glands.

Although the Meibomian glands of the asebic (*ab/ab*) males and females were present and secreting, they were much smaller than the glands of normal mice and the proportion of differentiating sebaceous cells was reduced. The increased acidophilia, typical of the most differentiated sebaceous cells in the pilosebaceous units of asebic mice, was also characteristic of the most differentiated sebaceous cells in these glands (Figs. 15, 16). Asebic mice from an early age seemed to suffer from sensitivity of the eyes to light, and scratching and inflammation of the eyelids was common. A sticky secretion was frequently visible to the naked eye, and eventually the mice appeared to become blind. However, these gross characteristics were more variable in their expression than the microscopically observed defects in the glands, which were constant for all Meibomian glands examined.


PLATE 3

Fig. 9. Electron micrograph of differentiating sebaceous cells from an *ab/ab* male, 8 days old. The SER system (arrows) is less extensive but intact in one cell, but disrupted in the adjacent cell to its lower right. Those lipid droplets which are present vary considerably in size. Uranyl acetate and lead citrate. × 11500.

Fig. 10. Part of a differentiating asebic sebaceous cell from an *ab/ab* male, 8 days old, showing a mitochondrion (*m*) containing a small dense granule, a bundle (*b*) of modified SER tubules and some lipid droplets (*l*). SER is less conspicuous in the cytoplasm of this cell than in the cells of + /*ab* mice (Plate 2). Uranyl acetate and lead citrate. × 31000.
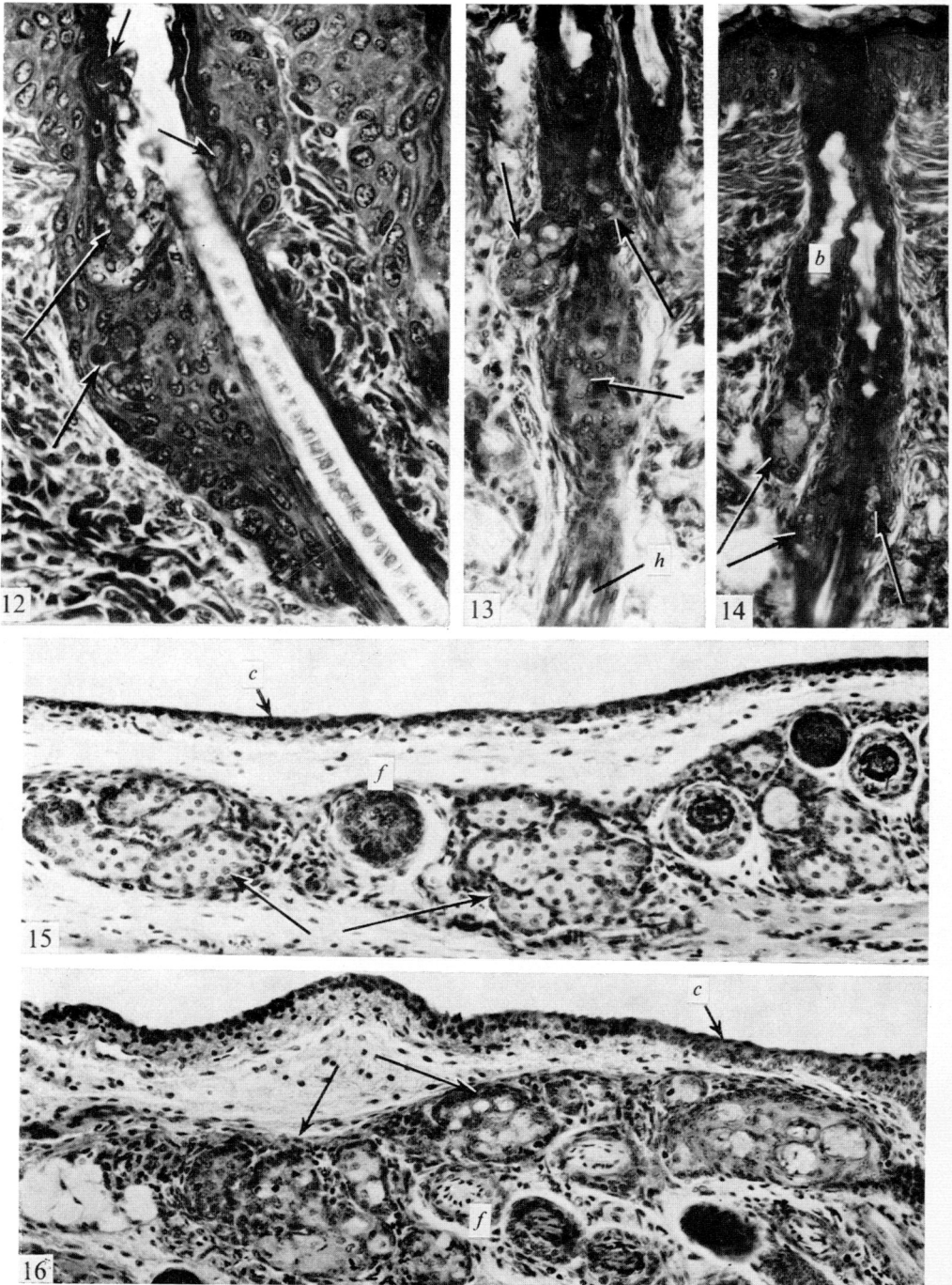
Fig. 11. A degenerating sebaceous cell from an *ab/ab* male. The disrupted tubule and droplet system is obvious, however some mitochondria (*m*) have clear cristae. Uranyl acetate and lead citrate. × 13000.
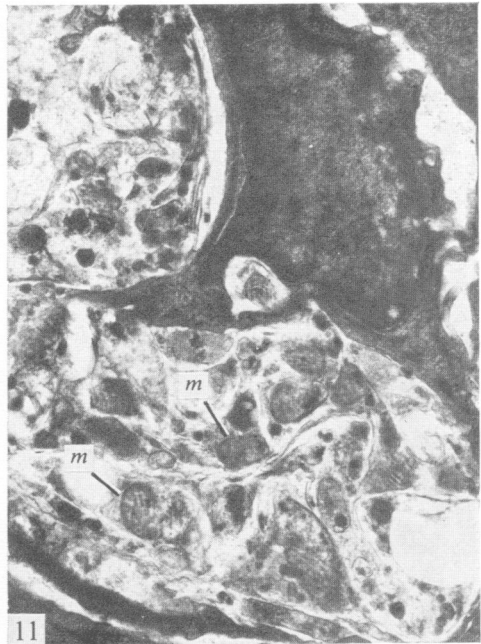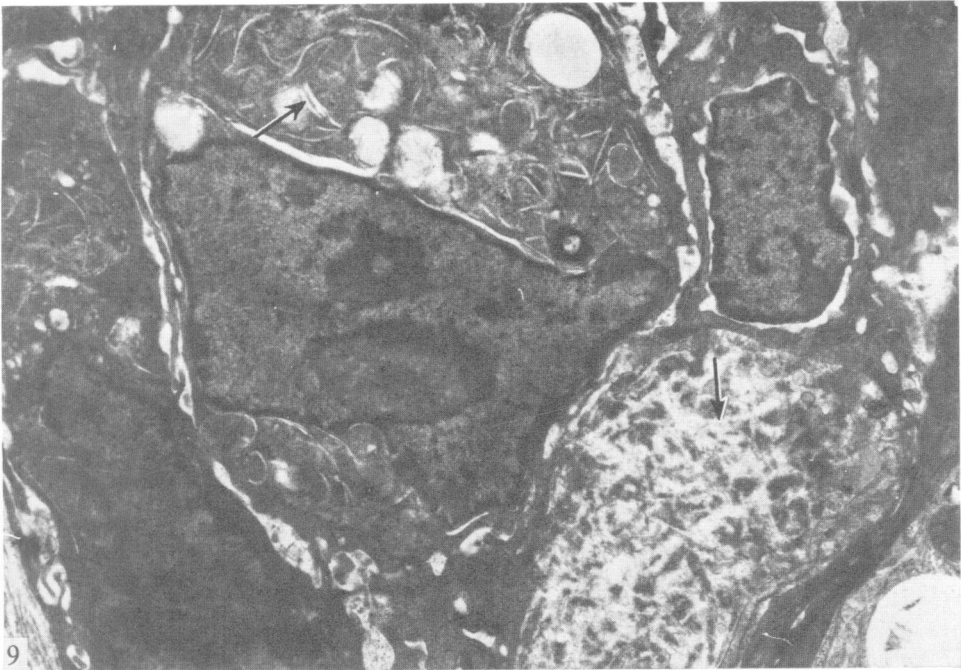
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While the leaf-shaped preputial glands and the anal sebaceous glands of the asebic male were apparently capable of normal sebaceous differentiation, the number of differentiating lobes was much lower than in normal males. No abnormality was seen in the clitoral glands or the anal glands of the young asebic females examined.

4. DISCUSSION

Gates & Karasek (1965) first described the *asebia* mutation in mice as one leading primarily to the absence of sebaceous glands. Nay (1972, 1973), using the BALB/cJ asebic strain, found that although *ab/ab* mice failed to form organized sebaceous glands, epithelial cells scattered within the upper level of the follicular ORS underwent atypical sebaceous transformation, as determined by their staining properties with Oil Blue N. However, in the BALB/cCrglGa strain asebic mice used in the present study, the sebaceous gland rudiments began as usual at 18 days post-conception and grew normally, and the defect was expressed by the abnormal sebaceous cytodifferentiation which began by the 20th day post-conception. We have found that actively secreting sebaceous glands, although small and differentiating abnormally, were a consistent feature of all follicles of asebic mice up to two years of age.

Morphologists are not agreed on the sequence of ultrastructural events which accompany sebum synthesis in the sebaceous glands of pelage follicles. The fact that species differ in the ultrastructure of their sebaceous cells (Montagna & Parakkal, 1974) makes it difficult to determine the significance of the changes observed in the differentiation of normal glands, let alone abnormal ones. However our observations on the ultrastructure of the glands of normal mice support the view of Rogers (1957) who implicated both the smooth endoplasmic reticulum (SER) and the mitochondria in the production and accumulation of sebum in the

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PLATE 4

Fig. 12. Asebic sebaceous gland from the mid-dorsal skin of an *ab/ab* male, 11 months old. The arrows indicate cells undergoing abnormal sebaceous differentiation. Mallory's triple connective tissue stain. × 275.

Fig. 13. A multilobed sebaceous gland associated with a hair follicle in the skin of an *ab/ab* male, 22 months old. The arrows point to areas of abnormal sebaceous differentiation. h – Hair shaft. Mallory's triple connective tissue stain. × 150.

Fig. 14. Asebic hair follicle found in the mid-dorsal skin of an *ab/ab* male, 22 months old. The arrows point to several areas of the follicle wall undergoing sebaceous differentiation. The follicle also has a separate and elongated sebaceous branch (*b*). Mallory's triple connective tissue stain. × 150.

Fig. 15. Meibomian glands of the eyelid from an + /*ab* male, 8 days old. The arrows point to the two clusters of sebaceous gland lobules associated with the central hair follicle (*f*). c – Conjunctival epithelium. The eyes do not open until 12 days after birth. Mayer's haemalum, eosin and picric acid. × 100.

Fig. 16. Meibomian glands of the eyelid of an *ab/ab* male litter-mate, 8 days old. The glands are smaller and the sebaceous cells are undergoing the same abnormal type of differentiation seen in the pelage follicle glands. The arrows point to the two clusters of sebaceous lobules associated with a hair follicle (*f*). Mayer's haemalum, eosin and picric acid. × 100.

glands of mice. As in normal mice, the sebaceous cells of asebic mice in the early phase of differentiation did possess both an extensive system of SER in the form of random undulating tubules and regular, closely packed bundles of modified tubules, and morphologically normal mitochondria. While in $+/ab$ glands further cellular differentiation was characterized by the controlled formation of uniform lipid droplets, during the differentiation of the asebic glands, the sebaceous cells fell into increased disarray. The distortion and dilation of the tubule system may be responsible for the reduction in number and uniformity of lipid droplets. The transformation of mitochondria which gradually took place in the differentiating sebaceous glands of normal mice was apparently not completed in the asebic glands.

Apparently the organelles required to initiate sebum production were relatively intact in the asebic sebaceous gland at the early stages of differentiation. The disruption which followed appeared to be due to defective regulation of the synthetic processes required for normal completion of sebaceous differentiation. Lysosomes, possibly originating from the bundles of modified SER tubules present in the sebaceous cells, have been considered responsible for the degenerative changes in normally differentiated sebaceous cells (Bell, 1969; Gutierriz & Aoki, 1973). The overproduction of lysosomes or the inability to prevent their release could also cause the apparent cellular damage and premature degeneration found in the asebic sebaceous glands.

The random differentiation of individual cells within the sebaceous gland, as well as the abnormal differentiation of nests of sebaceous cells from outer root sheath cells deep in the hypodermis, suggests the possibility of an abnormality in the initiation or induction of sebaceous differentiation within the outer root sheath cells. Of all the hair follicle components, the ORS, being nearest to the very abnormal dermis (Josefowicz & Hardy, 1978a, 1978b) would be expected to be most influenced by it.

While the Meibomian glands and the sebaceous glands of the pelage hair follicles were profoundly affected by the *asebia* mutation, two types of large specialized sebaceous glands, the anal glands and the glands of the external genital organ, remained fairly normal. The inductive and regulatory factors influencing the growth of the preputial glands are different from those of the sebaceous glands of pelage follicles. While the latter are apparently unaffected by progesterone, sebum production in the preputial glands is enhanced by progesterone in the presence of a 'sebotrophic' factor from the pituitary gland in rodents (Ebling, Ebling & Skinner, 1969a; 1969b). The specific and androgen-dependent nature of the mesenchyme required for preputial gland differentiation (Cunha, 1972a; 1972b) also distinguishes these glands from the sebaceous glands of pelage follicles. These differences seem to emphasize the significance of the role played by the adjacent mesenchymal tissue as well as the systemic hormonal environment in the induction, regulation and variability of sebaceous differentiation. The sebaceous gland abnormalities observed in asebic mice could result from defects related to these two influential factors.

Other integumentary abnormalities associated with the *asebia* mutation involving the epidermis and hair follicles were described previously (Josefowicz & Hardy, 1978*a*; 1978*b*). The hyperplasia of the epidermis and its appendages which became more apparent with age in the asebic mice, was characterized by the excessive thickness of the cellular epidermis, the uncontrolled differentiation of sebaceous cells within the ORS, the budding of hair follicles and ultimately the formation of metaplastic structures. Because of the gradual onset of these hyperplastic changes it seems unlikely that they are due to inherent defects within the pilosebaceous unit itself, but could be due to prolonged exposure to faulty dermal or systemic influences. Because differentiation and maturation (Sengel, 1975) as well as maintenance (Billingham & Silvers, 1967) of the epithelial structures in skin are directed by the adjacent mesodermal tissue, any dermal alterations could cause severe problems within the epidermal appendages. Since the epidermis, sebaceous glands and the follicular outer root sheath arise from the same tissue in embryonic life, and have many similarities, it is possible that in asebic mice all of the epidermal derivatives could become increasingly abnormal and hyperplastic with age as the result of a single source of persistent aggravation. Ebbesen's (1974) observations on non-mutant BALB/c mice suggest that with increasing age, and independent of age-related alterations in immune status or hormonal secretion levels, the skin cells themselves become more susceptible to carcinogens. Similarly, an altered dermal environment that initiated only minor abnormalities in the adjacent epidermal structures of young and growing asebic mice might have a more profound effect when epidermally derived cells become more sensitive with increased age.

The foregoing discussion suggests that the defects of hair follicle, sebaceous gland and epidermal growth, observed in mice homozygous for the *asebia* mutation, may not be inherent but may be secondary to, and variable with, primary changes in the dermis. Although dermal abnormalities may result from the direct action of the mutation, it is more probable that they may arise as a result of a single systemic abnormality. The fertility problems of the asebic mice, as well as their skeletal, adrenal and ovarian abnormalities (Josefowicz, 1975; Josefowicz & Hardy, unpublished observations) suggest the hypothesis that the *asebia* mutation does not act primarily on the skin but at a more basic endocrine or metabolic level. Some aspects of this hypothesis are currently being investigated.

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